

An In-Depth Analysis of Original Antigenic Sin in Dengue Virus Infection[▽]

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The evolution of dengue viruses has resulted in four antigenically similar yet distinct serotypes. Infection with one serotype likely elicits lifelong immunity to that serotype, but generally not against the other three. Secondary or sequential infections are common, as multiple viral serotypes frequently cocirculate. Dengue infection, although frequently mild, can lead to dengue hemorrhagic fever (DHF) which can be life threatening. DHF is more common in secondary dengue infections, implying a role for the adaptive immune response in the disease. There is currently much effort toward the design and implementation of a dengue vaccine but these efforts are made more difficult by the challenge of inducing durable neutralizing immunity to all four viruses. Domain 3 of the dengue virus envelope protein (ED3) has been suggested as one such candidate because it contains neutralizing epitopes and it was originally thought that relatively few cross-reactive antibodies are directed to this domain. In this study, we performed a detailed analysis of the anti-ED3 response in a cohort of patients suffering either primary or secondary dengue infections. The results show dramatic evidence of original antigenic sin in secondary infections both in terms of binding and enhancement activity. This has important implications for dengue vaccine design because heterologous boosting is likely to maintain the immunological footprint of the first vaccination. On the basis of these findings, we propose a simple *in vitro* enzyme-linked immunosorbent assay (ELISA) to diagnose the original dengue infection in secondary dengue cases.

Dengue virus is an insect-borne flavivirus transmitted to humans by the bite of an infected mosquito, usually *Aedes aegypti* (20). There are four circulating serotypes of dengue (dengue serotype 1 [Den1] to Den4) that show up to 70% sequence homology across their genomes (4, 17), and it is common for multiple viral serotypes to cocirculate in countries where dengue is endemic. Most dengue infections are either asymptomatic or lead to uncomplicated dengue fever (DF). However, in 1 to 5% of cases, symptoms can be more severe with the development of plasma leakage and hemorrhage. Such dengue hemorrhagic fever (DHF) can lead to circulatory collapse, resulting in a mortality rate of around 20% if left untreated.

The more frequent occurrence of DHF in secondary dengue infections in children and adults suggests a role for the acquired immune system in disease pathogenesis, and there has been considerable research into both the B- and T-cell responses. Antibody-dependent enhancement (ADE) of infection, proposed by Halstead in 1977 (24, 25), is one hypothesis for this increase in severity in secondary infections (23, 36). During a primary infection, antibodies that cross-react with the remaining 3 serotypes are induced. After a few months, when heterologous protection is no longer observed (54), it is hypothesized that these cross-reactive antibodies decline to sub-neutralizing levels, meaning that a heterologous infecting serotype is not controlled. Antibody made against the primary infecting virus may not be of sufficient avidity to neutralize a second serotype. Instead, these poorly neutralizing, low-avidity cross-reactive antibodies bind the secondary virus and target it to Fcγ receptor-bearing cells, such as macrophage/monocytes (24, 25), leading to internalization and increased virus replication. *In vivo*, ADE has been shown to induce lethal disease in mice (2) and to drive high virus loads in primates (16, 22).

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ADE has also been invoked to explain a peak in disease severity in primary cases during the first year of life, as the titers of passively transferred maternal antibody fall (23, 35, 41, 56).

The incidence of dengue increased sharply in the middle of the last century and is still increasing at an alarming rate (20). There are estimated to be around 3.6 billion people living in the tropics and subtropics who are at risk from dengue, with up to 50 million predicted infections per annum. To date, however, there are no specific treatments for dengue barring careful attention to fluid replacement. The scale of the problem posed by dengue has spawned much interest in the development of a dengue vaccine, with some candidates in phase II trials (67, 69), and also in anti-dengue drugs that have not yet reached clinical trials.

A number of vaccine candidates, ranging from live attenuated viruses to subunit vaccines, are currently being pursued. The envelope (E) protein of dengue is a major target of neutralizing (48, 53, 62) and protective antibodies (32) and, as such, should be a key component for any subunit vaccines. The envelope protein consists of three domains: ED1, ED2, and ED3 (38, 47, 48). ED3 is proposed to be the binding domain for the virus (7, 9, 28), attaching to as yet poorly characterized cellular receptor(s); although heparan sulfate has been implicated in the interaction (29). Indeed, in mice, anti-ED3 monoclonal antibodies are potent neutralizers of dengue virus (5, 18, 19, 27, 43, 52, 55, 60, 61); often neutralizing to greater levels than those targeting ED1 or ED2 (60). ED3 is a target of both serotype-specific (5, 18, 42, 45, 53, 55, 60, 61) and cross-reactive (19, 43, 46, 52, 55, 60, 61) neutralizing antibodies. Of the anti-ED3 antibodies that are strongly neutralizing, however, the majority are usually serotype specific (5, 55, 60), and cross-reactive antibodies are generally weaker neutralizers (19, 61). There have been a number of vaccination studies investigating ED3 as a potential immunogen (3, 30, 57–59).

In this study, we have performed a detailed analysis of the anti-ED3 antibody responses in humans, using a cohort of patients experiencing either primary or secondary dengue infections. We demonstrate that low-level dengue cross-reactive anti-ED3 responses are induced upon primary infection and boosted dramatically during a secondary heterotypic infection. We have developed a competition enzyme-linked immunosorbent assay (ELISA) to measure the relative avidities of these cross-reactive antibodies and show that the response is dominated by original antigenic sin. We propose this ELISA as a simple diagnostic test for determining the serotype of the original dengue infection in secondary dengue cases. Furthermore, we go on to show an inverse relationship between the avidity of the antibody response and enhancing activity.

MATERIALS AND METHODS

Plasma samples. Blood samples were collected, following written parental consent and the approval of the ethics committee at the Khon Kaen Hospital in Thailand and the Riverside Ethics Committee in the United Kingdom. Plasma samples were separated from the blood samples, transported to the United Kingdom, and stored at -80°C prior to use. Individuals diagnosed with dengue fever (DF) ($n = 19$) or different stages of dengue hemorrhagic fever (DHF) ($n = 52$) following infection with different serotypes were analyzed. Individuals were aged 4 to 14 years, with approximately 57% male and 43% female. During the course of this study, we acquired 4 additional pairs of samples from patients in Thailand (K1 to K4), for whom we had samples both before and after secondary exposure. Of these 4 patients, 2 individuals were experiencing DF and 2 indi-

viduals were experiencing DHF. Plasma samples ($n = 34$) were also analyzed from Vietnamese pediatric dengue patients experiencing DF; these samples were collected following written parental consent as part of an ongoing prospective study of dengue at two primary health care clinics in Ho Chi Minh City, Viet Nam. The study protocol was approved by the Hospital for Tropical Diseases and the Oxford Tropical Research Ethical Committee. The current infecting serotype was determined by reverse transcription-PCR (RT-PCR)-based gene identification (71). Secondary dengue infections were defined by a dengue-specific IgM/IgG ratio of <1.8 by IgM and IgG capture ELISA.

ED3 expression and purification. ED3 (amino acids [aa] 295 to 401) of the E protein of each of the four dengue serotypes was expressed and purified for use in ELISA. Prototype strains of each serotype were used: Den1 strain Hawaii, Den2 strain 16681, Den3 strain H87, and Den4 strain H241. The PCR-generated ED3 inserts were cloned into a bacterial expression vector, pET3c (Novagen), for expression under isopropyl- β -D-thiogalactopyranoside (IPTG) induction. ED3 of serotype 1 was expressed in Rosetta competent cells (Novagen), ED3 of serotype 2 was expressed in Origami competent cells (Novagen), and ED3 of serotypes 3 and 4 was expressed in BL21(DE3) competent cells (Novagen) (all cells are *Escherichia coli*). ED3 proteins are expressed in inclusion bodies (IBs), which were washed 5 times by Dounce homogenization in 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 1% Triton X-100. Between each wash, the suspension was clarified by centrifugation ($10,000 \times g$, 30 min, 4°C , Heraeus biofuge JA-20R). IBs were denatured in 100 mM Tris-HCl, 6 M guanidine-HCl, and 2 mM dithiothreitol (DTT) and left overnight at 4°C . The lysate was clarified by centrifugation ($15,000 \times g$, 25 min, 4°C , Heraeus biofuge 15R), and the supernatant was collected for refolding. The denatured protein (50 mg) was then added to 500 ml of refolding buffer (100 mM Tris-HCl, 500 mM L-arginine-HCl, 0.2 mM EDTA, 3.7 mM cystamine, 100 μM phenylmethylsulfonyl fluoride [PMSF], 6.6 mM β -mercaptoethylamine) over a period of 2 h via a dropwise method and left spinning at 4°C for 48 h. Refolded protein was concentrated, buffer exchanged into phosphate-buffered saline (PBS), and purified in PBS through a size exclusion column (26/60 Superdex 75; GE Healthcare). Protein was quantified by calculation with extinction coefficient, by BCA protein kit (Pierce, United Kingdom), and by Bio-Rad protein assay.

ED3 direct ELISA. Purified ED3 from serotypes 1, 2, 3, and 4 [ED3(1, 2, 3, and 4)] and bovine serum albumin (BSA) were used as coating antigen and negative-control antigen, respectively. Ninety-six-well plates were coated overnight at 4°C with 150 ng protein in 50 μl of coating buffer (50 mM NaHCO_3 , pH 9.6). The plates were rinsed 3 times with PBS containing 0.1% Tween 20, using an automated 96-well plate washer, and were blocked for 2 h at room temperature (RT) with 200 μl of blocking buffer (PBS containing 1.5% BSA). The plates were incubated for 90 min with 50 μl of 3-fold serial dilutions of sample plasma in dilution buffer (PBS containing 0.1% Tween 20 and 0.5% BSA) and then for 90 min at RT with 50 μl of alkaline phosphatase-conjugated anti-human IgG secondary antibody (Sigma-Aldrich) (1:10,000 in dilution buffer). The activity was observed with *p*-nitrophenyl phosphate substrate (Sigmafast; Sigma-Aldrich) (optical density at 405 nm [OD_{405}]) and measured with Magellan Plate reader software. ELISA IgG endpoint titers (EPTs) were defined as reciprocal plasma dilutions corresponding to 2 times the average OD values obtained with an irrelevant antigen (BSA). Pooled convalescent-phase serum (PCS), obtained from recovering dengue patients, was used to standardize EPTs between plates and assays. To adjust for antigen loading on the plates, EPTs were also normalized to the saturation plateaus of monoclonal antibody (MAb) 2H12 in the ED3 ELISA. These were OD_{405} values of 2.09, 2.03, 1.87, and 1.95 for ED3 of serotype 1 [ED3(1)], ED3(2), ED3(3), and ED3(4), respectively. The seropositivity cutoff values for these 4 serotype-specific assays were defined as $3 \times$ geometric mean titer (GMT) of dengue-naïve serum ($n = 20$). These values were 1,282, 938, 907, and 837 for ED3(1), ED3(2), ED3(3), and ED3(4), respectively, and each gave 100% specificity. Any EPT less than the cutoff value was subsequently given a “negative” value of half the cutoff value.

Dissociation constants of monoclonal antibody 2H12. An ED3 direct ELISA was carried out with MAb 2H12, ensuring that saturation plateaus were reached. The assay was carried out as described above except that an alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich) (1:10,000 in dilution buffer) was used. For each antigen, the percent maximum OD was calculated and the dissociation constants (K_d) values were determined (Prism Software).

ED3 competition ELISA. Purified ED3(1, 2, 3, and 4) and BSA were used as coating antigen and negative-control antigen, respectively. Ninety-six-well plates were coated overnight at 4°C with 150 ng protein in 50 μl of coating buffer (50 mM NaHCO_3 , pH 9.6). The plates were rinsed 3 times with PBS containing 0.1% Tween 20, using an automated 96-well plate washer, and were blocked for 60 min at 37°C with 200 μl of blocking buffer (PBS containing 1.5% BSA). During this

incubation step, plasma samples were incubated with 10-fold serial dilutions of competing antigen in a separate 96-well plate for 60 min at 37°C. All dilutions were made in dilution buffer (PBS containing 0.1% Tween 20 and 0.5% BSA). For each antigen coated, one concentration of plasma was chosen (1 in 300 to 1 in 2,000). This was determined by the anti-ED3 (coated-antigen) EPT, such that a similar OD was observed for all 4 ED3 serotypes in the absence of competing antigen. Each plasma sample was competed with each of the 5 competing antigens separately: ED3(1), ED3(2), ED3(3), and ED3(4) and FasD1 as the negative control. ELISA plates were then incubated for 60 min with 50 μ l of the plasma/competing antigen mixture and for a further 60 min at 37°C with 50 μ l of alkaline phosphatase-conjugated anti-human IgG secondary antibody (Sigma-Aldrich) (1:5,000 in dilution buffer). The activity was observed with *p*-nitrophenyl phosphate substrate (Sigmafast; Sigma-Aldrich) (OD_{405}) and measured with Magellan Plate reader software. The background response against BSA in the absence of competing antigen was deducted from each of the responses obtained. The percent maximum OD for each coated antigen was then calculated, using the response in the absence of competing antigen.

Focus reduction neutralization test. Micro-focus reduction neutralization tests (micro-FRNTs) were conducted on Vero cells as described previously (31). Den1 strain Hawaii, Den2 strain 16681, Den3 strain H87, and Den4 strain H241 were used. The FRNT₅₀ titer was defined as the reciprocal plasma dilution that reduced the number of foci by 50%.

Enhancement assay on U937 cells. Den1 strain Hawaii and Den2 strain 16681 were used in these assays. Supernatants from Den1- or Den2-infected cells were incubated with 3-fold serial dilutions of plasma, in a 96-well U-bottom plate, for 30 to 60 min at 37°C. Plasma samples were heat inactivated at 56°C before use. The 2 virus stocks were equated for this assay by choosing the concentrations of virus that gave overlapping enhancement peaks with PCS. Supernatant from mock-infected cells was used as a negative control. All dilutions were made in R2: RPMI 1640 (Gibco) supplemented with 2% heat-inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine (Invitrogen), 50 units/ml penicillin, and 50 μ g/ml streptomycin (Invitrogen). Meanwhile, U937 cells were plated in 96-well U-bottom plates (150,000/well in 30 μ l). Fifty microliters of the virus-plasma mixture was then added to the cells and incubated at 37°C. After 2 h, an additional 120 μ l of R10 (RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), 2 mM L-glutamine (Invitrogen), 50 units/ml penicillin, and 50 μ g/ml streptomycin (Invitrogen)) was added and the cells were left to incubate for a further 18 h. The cells were then stained intracellularly (see section below) for analysis by flow cytometry. The percentage of infected cells was recorded, and the percentage of infection observed for each virus in the absence of antibody (<2%) was deducted.

Monocyte infection assay. Supernatant from Den2 strain 16681 virus-infected cells, resulting in an MOI of 4 upon infection, was incubated with 2-fold serial dilutions of PCS, in a 96-well U-bottom plate, for 30 to 60 min at 37°C. The PCS was heat inactivated at 56°C before use. Supernatant from mock-infected cells was used as a negative control. All dilutions were made in R10. Meanwhile, freshly isolated peripheral blood mononuclear cells (PBMCs) were plated in 96-well U-bottom plates (150,000/well) and centrifuged (400 \times g, 5 min). Fifty microliters of the virus-plasma mixture was then added to the PBMC pellet and incubated at 37°C. After 2 h, an additional 150 μ l of R10 was added, and the cells were left to incubate for a further 18 h. The cells were then stained intracellularly (see section below) for analysis by flow cytometry. Monocytes were gated by forward and side scatter, and the percentage of infected cells was recorded.

Intracellular staining of infected cells. Infected U937 cells or PBMCs were transferred to a 96-well V-bottomed plate and washed twice with PBS by centrifugation at 700 \times g for 2 min at 4°C. The pellet was then fixed with 100 μ l of 5% formaldehyde for 10 min at RT and washed in PBS. The cells were then permeabilized for 10 min at RT in 100 μ l of fluorescence-activated cell sorting (FACS) wash (PBS containing 0.5% saponin, 0.5% BSA, 1% FBS, 0.5% human serum, 0.1% sodium azide), and washed again in FACS wash. The cells were then incubated with 50 μ l of MAb 4G2 (10 μ g/ml in FACS wash) for 45 min at 4°C. The cells were washed twice, and the pellet was subsequently incubated in 50 μ l of phycoerythrin (PE)-conjugated anti-mouse immunoglobulins (5 μ g/ml in FACS wash; Dako) for 30 to 45 min at 4°C. The cells were then washed twice more in FACS wash and a third time in PBS before analysis by flow cytometry (FACSCalibur; Becton Dickinson).

Depletion of anti-ED3 antibodies from plasma samples. Nickel charged agarose beads (nickel-nitrilotriacetic acid [Ni-NTA]; Qiagen) were washed 5 times in PBS⁺ (PBS containing an additional 150 mM NaCl and 50 mM imidazole) and then incubated overnight, rotating at 4°C, with C-terminally His-tagged ED3(1) and ED3(2) mixed together. Ten micrograms of each protein was added for every 50 μ l of beads used in a final incubation volume twice the bead volume. Some beads were incubated in the absence of ED3 antigen as a beads-only, mock

control. The beads were then washed 5 times with PBS⁺ and precleared for 2 h at RT with a dengue-naïve human serum sample at a dilution of 1 in 100 in an incubation volume 5 times the bead volume. Beads were then washed 3 times in PBS⁺ and incubated with the human plasma samples of interest (50 μ l beads per 800- μ l sample) for 2 h at 4°C. The convalescent-phase samples (days 15 to 30 after defervescence) were diluted in PBS⁺ (primary infections, 1 in 250; secondary infections, 1 in 400) before mixing with the beads. The remaining depleted samples were collected, filter sterilized, and tested for complete depletion by ED3 direct ELISA.

RESULTS

The anti-ED3 response following primary infections. With much of the recent interest in ED3 as a possible dengue vaccine immunogen, we initially set out to determine the anti-ED3 response in humans. Convalescent blood samples were collected from individuals 2 to 4 weeks following their presentation to hospital with dengue infections. We first examined anti-ED3 IgG binding responses by direct ELISA against bacterially expressed recombinant ED3 in individuals convalescing from a primary dengue infection (Fig. 1). To account for the amount of each serotype antigen coated on the wells of the ELISA plates, we normalized our responses with an anti-ED3 monoclonal antibody, 2H12, generated in our laboratory. This is highly cross-reactive between the four serotypes, exhibiting mean ($n = 4$) K_d values of 0.4496, 0.4248, 0.4655, and 0.4215 nM for ED3(1), ED3(2), ED3(3), and ED3(4), respectively. Saturation binding of MAb 2H12 to the coated wells of the ELISA plates varied by less than 11%, and titers were normalized against these saturation OD values to allow for a fair comparison between serotypes. We also have confidence in the refolding of these antigens, as the same material was used to obtain crystal structures of ED3 proteins from dengue serotype 1 (Den1), Den2, and Den3 complexed with the Fab fragments of three separate anti-ED3 monoclonal antibodies, showing the correct ED3 conformation (our unpublished data).

In the primary dengue cases, the strongest anti-ED3 responses were elicited during Den1 infections, and the responses following Den2 and Den3 infections were lower (Fig. 1a). Across all infections, however, the highest response detected in most individuals was against the infecting serotype. This is consistent with previous reports, whereby a serotype-specific ED3 response was described after primary infection (44, 66). However, we also detected low yet positive responses against the remaining 3 serotypes, demonstrating a degree of cross-reactivity. We established a competition assay to investigate this cross-reactivity in more detail (Fig. 1b and c). Immune plasma samples were incubated with an increasing concentration of recombinant ED3 from the four virus serotypes or with a control protein. Following incubation, the mix was added to the wells of ELISA plates (wells coated with ED3), and the level of bound anti-ED3 antibodies, representing those not competed, was determined. Responses could be ranked in terms of avidity, with the highest avidity being displayed by the antigen that was able to compete at the lowest concentration.

Taking an individual with a primary Den1 infection (Fig. 1b), the ED3(2)-specific response could be completely blocked by the presence of competing ED3(1) protein. Conversely, when the plate was coated with ED3(1), ED3(2) was a poor competitor compared with ED3(1). This shows very clearly that the ED3(2)-specific response was indeed present and fur-

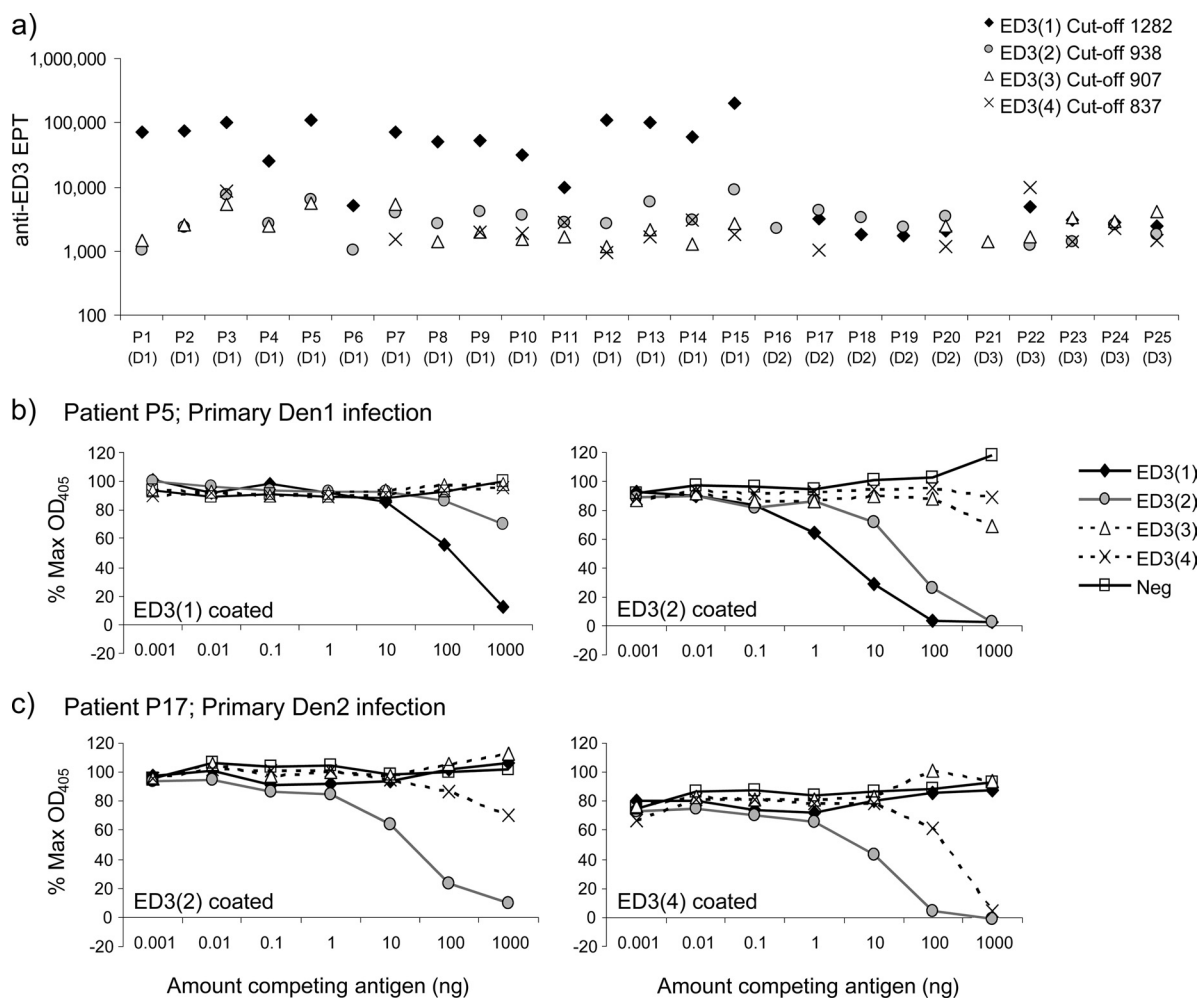


FIG. 1. Serotype cross-reactive anti-ED3 antibodies are induced after primary dengue infections in humans. (a) Anti-ED3 endpoint titers (EPTs) were determined for all 4 serotypes by direct ELISA against bacterially expressed recombinant ED3. All plasma samples taken 15 to 30 days after defervescence from individuals with primary dengue infections are shown ($n = 25$). The cutoff levels of seropositivity for each assay are indicated in the symbol key. Assay values below these cutoffs are not depicted, meaning that for some individuals, not all 4 titers are shown. Twenty-five plasma samples from individuals with primary infections (P1 to P25) are shown below the x axis. The current infecting serotype (Den1 [D1] to Den4 [D4]) as determined by PCR is shown in parentheses below the plasma sample. (b) Competition ELISA for an individual with primary Den1 infection. The anti-ED3(1) and anti-ED3(2) responses, for which ED3(1) and ED3(2) are coated on the ELISA plate, respectively, are shown. The percent maximum OD₄₀₅ is shown on the y axes. Neg, negative. (c) Competition assay for an individual with primary Den2 infection. The anti-ED3(2) and anti-ED3(4) responses are shown.

thermore was completely cross-reactive with dengue serotype 1. Importantly, ED3(1) protein could completely block the response with 100-fold less antigen than competing with ED3(2) protein, indicating a higher avidity to the infecting serotype. The same effect is illustrated with a second patient (Fig. 1c). Following a primary infection with Den2, an ED3(4)-specific response was detected and could be completely inhibited by the presence of ED3(2) antigen. Again, the plasma avidity was strongest against the infecting serotype, a trend seen with the primary samples tested ($n = 10$).

Complete cross-reactivity in secondary infections. Anti-ED3 analyses of convalescent plasma samples from individuals experiencing secondary infections revealed that both the serotype-specific and cross-reactive antibody responses were higher than those following primary infection, with endpoint titers reaching 480,000 (Fig. 2). Most individuals were seropositive

for all 4 ED3 antigens, but unlike the situation in primary infections, the titers gave no clear indication as to what the current or previous infecting serotypes may have been. In fact, titers against ED3(1) seemed to be slightly higher than the other serotypes, regardless of the infecting serotype.

Next, we went on to perform the competition ELISA in secondary infection; two representative cases are shown (Fig. 2b and c). Following a secondary infection with Den1, the ED3(1) response could be completely inhibited by competition with ED3(2) (Fig. 2b). Similarly cross-reactive responses to the other 3 serotypes were also completely inhibited by ED3(2). A similar pattern is seen with a patient suffering a secondary Den2 infection (Fig. 2c). In this case, ED3(1) could completely inhibit binding to all four virus serotypes. The anti-ED3 response in these secondary infections was 100% cross-reactive, and this was true for all samples tested ($n = 30$). It was also

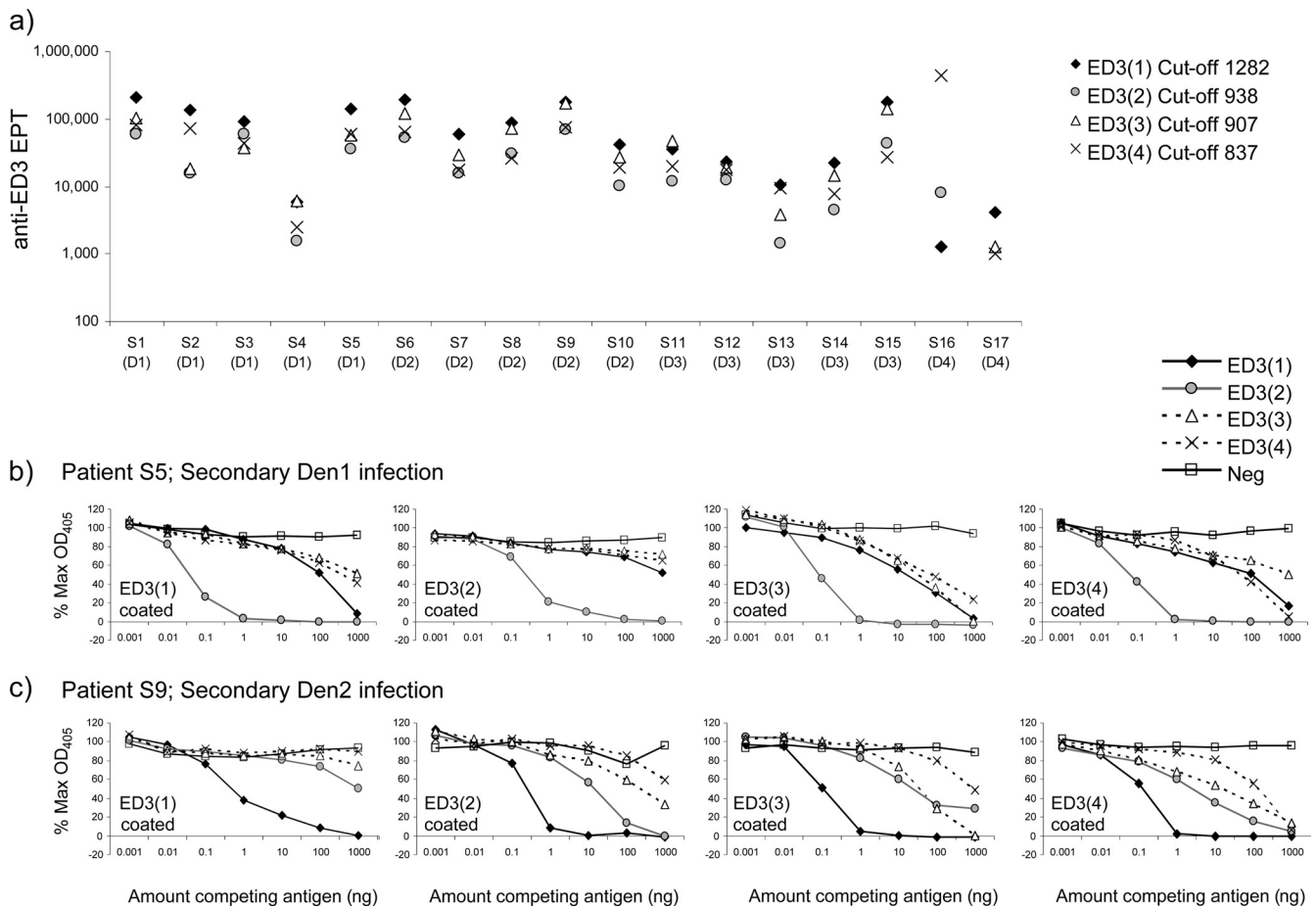


FIG. 2. Anti-ED3 antibodies are completely cross-reactive in secondary dengue infections in humans. (a) Anti-ED3 endpoint titers (EPTs) were determined for all 4 serotypes by direct ELISA against bacterially expressed recombinant ED3. A representative selection of plasma samples, taken 15 to 30 days after defervescence from individuals with secondary dengue infections, is shown ($n = 17$ out of 76 analyzed). The cutoff levels of seropositivity for each assay are indicated in the symbol key. Assay values below these cutoffs are not depicted, meaning that for some individuals not all 4 titers are shown. Seventeen plasma samples from individuals with secondary infections (S1 to S17) are shown. The current infecting serotype (D1 to D4), as determined by PCR is shown. (b) Competition ELISA for an individual with secondary Den1 infection. The responses against all 4 ED3 serotypes are shown. (c) Competition assay for an individual with secondary Den2 infection. The responses against all 4 ED3 serotypes are shown.

always the case, barring one sample, that antigen from a serotype that was not that of the current infecting serotype provided the best competition. Up to 10,000-fold-less antigen was required to outcompete the response against the infecting serotype, indicating a significantly stronger avidity to this other serotype. Interestingly, the strong binding responses seen against ED3(1) were not reflected in antibody avidity, and instead all serotypes were identified as “winners” at least once.

Dramatic original antigenic sin in secondary dengue infections. Original antigenic sin was first described for the antibody responses following sequential influenza infections (10, 13–15, 68). Rather than effective *de novo* neutralizing responses being elicited against the current infecting strain, memory responses to the primary infecting strain were induced. Halstead showed that original antigenic sin was also present in the neutralizing antibody responses to dengue (26). In line with this, our competition results, in which 100% of antibody produced to the secondary infecting virus showed cross-reaction and higher

avidity to a different serotype, suggested the presence of original antigenic sin in these secondary anti-ED3 responses.

In order to test this hypothesis, we investigated samples from secondary infections in which the primary exposure was known. Pre- and postexposure plasma samples were available from individuals experiencing a secondary dengue infection. In cases where a clear primary dengue serotype could be identified from neutralization analyses of the preexposure sample, a competition assay was performed on the postexposure convalescent samples (Fig. 3). In each case, the serotype with the highest avidity in the competition assay matched the primary exposure identified by neutralization analyses. For example, with patient K1, the FRNT₅₀ of the preexposure plasma sample strongly indicated that Den1 had been the primary infection (Fig. 3a). In agreement with this, when we tested the postexposure plasma samples in the competition assay, protein from serotype 1 was able to completely outcompete all responses at the lowest concentrations (Fig. 3b).

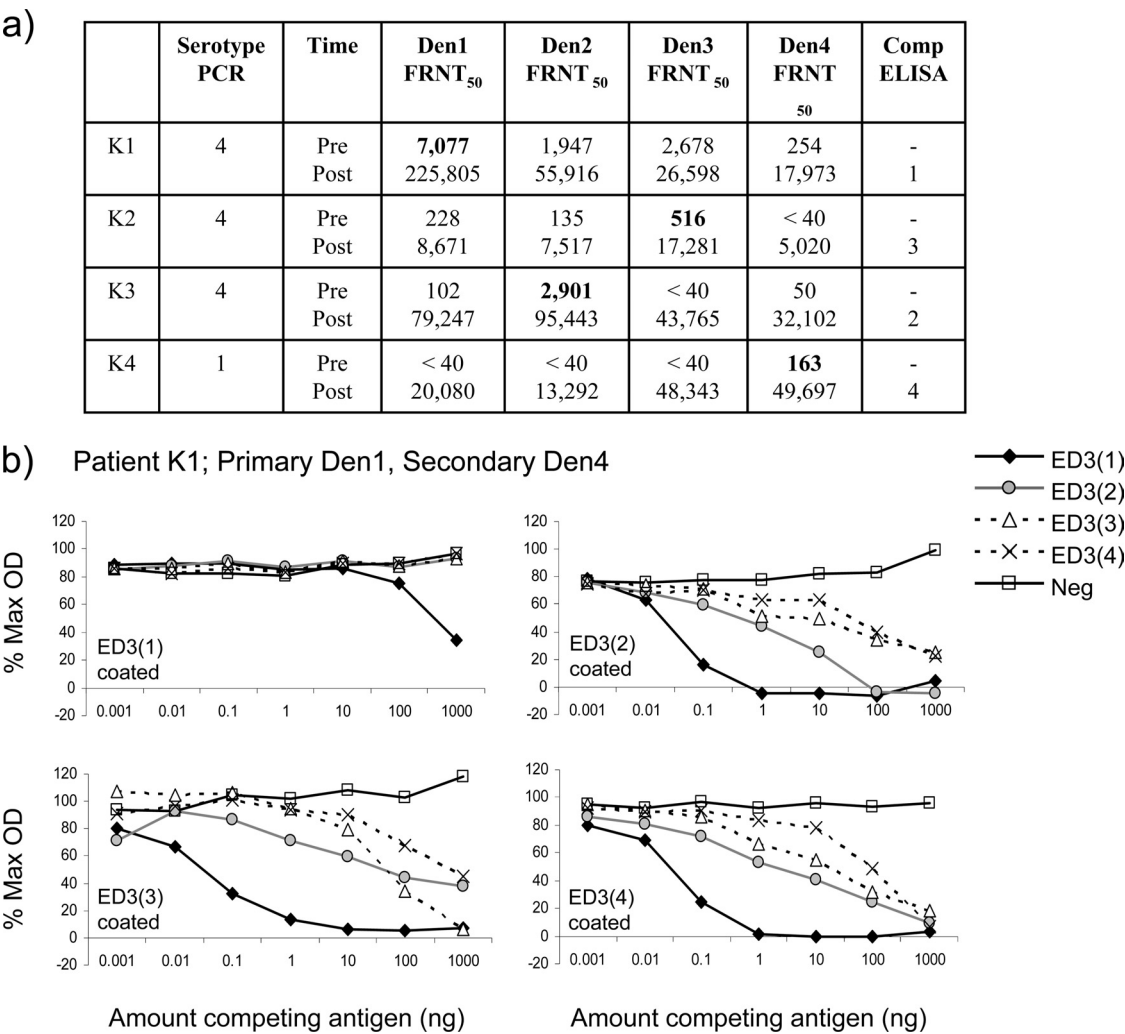


FIG. 3. ED3 competition assay predicts the primary infecting serotype. (a) The pre- and postexposure plasma samples from a selection of individuals with secondary infections ($n = 4$) were analyzed for neutralizing activity, and this information was used to determine the primary infecting serotype (boldface values). A competition ELISA (Comp ELISA) was then carried out on the corresponding 2- to 4-week postexposure plasma samples. (b) Competition ELISA for a single individual (K1) with a secondary Den4 infection who had a primary Den1 exposure. The responses against all ED3 serotypes are shown.

In 1983, Halstead proposed that original antigenic sin in the postexposure neutralization response could help to deduce the serotype of the primary infecting virus in that the strongest neutralizing activity is generally directed toward the primary infecting serotype (26). It is often thought that in either an acute or convalescent sample, the serotype with the highest 50% neutralizing dose (ND₅₀) is indicative of the primary infecting serotype. There are different interpretations for this type of diagnosis (12, 26, 39), but a titer of at least 4-fold that of other serotypes would provide a highly stringent diagnosis.

We performed focus reduction neutralization tests (FRNTs) on a selection of paired immune plasma samples from acute and convalescent time points, and the FRNT₅₀s were compared to the results from the competition ELISA (Table 1). For individuals in whom one serotype exhibited a 4-fold-higher FRNT₅₀, and thus the primary exposure was clear ($n = 10$) (Table 1), the competition assay did indeed predict the primary infecting serotype. Unfortunately, it is often the case that neu-

tralizing responses are complex, with neutralization of multiple viruses occurring. This does not always allow for the clear determination of the primary infecting serotype (1, 39). In our study, in individuals in whom one serotype did not exhibit a 4-fold-higher FRNT₅₀ than the others ($n = 5$) (Table 1), the competition ELISA was successful in indicating a probable primary serotype. As a result, this quick, easy, virus-free competition assay could provide an alternative to the time-consuming neutralization assay in current use.

Antibody-dependent enhancement in primary and secondary dengue infections. Original antigenic sin in the antibody response to dengue has two potential adverse outcomes for the infected host. First, by driving the production of low-avidity antibodies, viral clearance may be delayed. Second, original antigenic sin has the potential to affect antibody-dependent enhancement (ADE). At present, ADE is proposed to be driven predominantly by preexisting antibody. However, as there are substantial increases in antibody during the early

TABLE 1. FRNT₅₀ titers and competition ELISA results from secondary infections

Patient ID ^a	Clinical diagnosis ^b	Serotype PCR ^c	Time ^d	FRNT ₅₀ titer to ^e :				Comp ELISA ^f
				Den1	Den2	Den3	Den4	
S1	DHF1	1	A C	43,174 16,542	465,764 147,704	7,868 1,950	14,974 5,806	2
S4	DHF2	1	A C	267 215	95 103	1,551 2,238	10 10	3
S5	DHF2	1	A C	980 13,482	13,240 110,122	528 6,084	112 1,128	2
S6	DHF1	2	A C	2,010 2,560	201 2,560	135 2,560	10 167	1
S9	DHF1	2	A C	1,639,400 65,804	494,864 84,338	356,369 24,658	109,436 28,336	1
S28	DHF1	1	A C	904 1,087	457 1,001	2,167 2,560	13 28	3
S38	DHF2	1	A C	1,318 3,921	6,728 55,032	1,095 3,944	6,718 8,003	2
S39	DHF2	1	A C	43,880 24,900	110,262 47,524	3,530 2,044	9,854 3,194	2
S41	DHF3	1	A C	128,691 25,120	502,032 61,196	74,129 8,861	251,381 37,808	2
S44	DHF3	1	A C	482 734	202 446	2,227 2,560	100 74	3
S45	DHF1	1	A C	20,702 29,754	98,716 22,868	3,466 8,212	2,476 2,714	2
S46	DHF3	1	A C	5,252 2,984	56,650 18,976	902 494	2,202 516	2
S54	DHF1	2	A C	17,182 107,270	6,476 47,328	548 2,554	712 3,302	1
S64	DHF3	2	A C	5,769,072 399,288	615,852 295,067	426,661 36,070	183,843 85,400	1
S65	DHF1	2	A C	2,560 2,560	531 537	1,132 1,261	38 54	1

^a Patient ID, patient identification.^b DHF1 to DHF3, increasing grades of dengue hemorrhagic fever severity, as designated by the WHO criteria.^c Serotype PCR, original PCR diagnosis of current infection. 1 and 2, Den 1 and Den 2, respectively.^d A, acute phase; C, convalescent phase.^e The FRNT₅₀ titer was defined as the reciprocal plasma dilution that reduced the number of foci by 50%. Titers that are >4-fold that of other serotypes are shown in boldface type.^f The serotype with the highest avidity in the competition ELISA (Comp ELISA) is listed.

stages of secondary infection, boosting of poorly neutralizing, low-avidity responses during the course of the secondary infection may amplify this effect still further.

We therefore examined ADE in patients following either primary or secondary infection. ADE assays were performed on U937 cells comparing infection with either Den1 or Den2, and the percentage of infected cells was recorded (Fig. 4). Months after a primary infection with Den1, at a point when individuals would be expected to become susceptible to infections with heterologous serotypes, the peaks of enhancement for Den2 occurred at higher concentrations of plasma than Den1 (Fig. 4b), indicating that enhancement of Den2 was

more likely. We next examined two scenarios of secondary infection: Den1 followed by Den2 (Fig. 4c) and Den2 followed by Den1 (Fig. 4d). In each case, the concentration of immune serum required to enhance the second virus was greater than the concentration required to enhance the first virus, a pattern similar to that observed with the primary immune sera. This indicates that an individual would likely be more susceptible to the secondary virus, as has been reported previously in epidemiological studies (23). Furthermore, if we follow the time course of a secondary Den2 infection, the plasma continues to enhance Den2 more readily throughout (Fig. 4e). These data demonstrate that there is an inverse

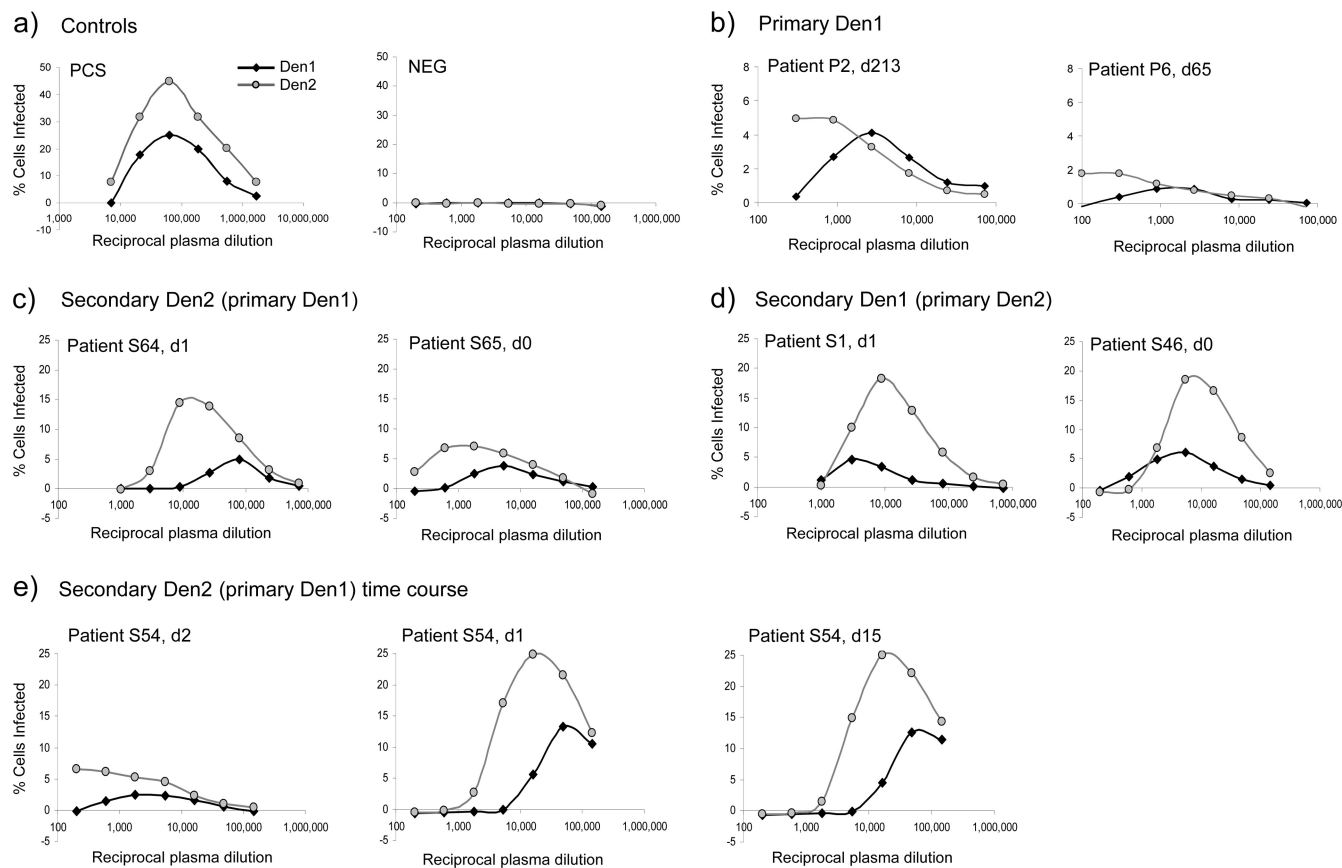


FIG. 4. Antibody-dependent enhancement (ADE) in primary and secondary dengue infections. Enhancement assays of Den1 and Den2 were performed on U937 cells. (a) The two virus stocks were equated by aligning the peaks of pooled convalescent-phase serum (PCS) enhancement. Virus was incubated for 1 h at 37°C with decreasing concentrations of serum/plasma and then added to U937 cells for a further 24 h. The cells were stained intracellularly with the dengue cross-reactive monoclonal antibody 4G2. The percentage of infected cells was determined by flow cytometry, and the background levels (in the absence of plasma) were deducted. (b to d) Enhancement assays were carried out using plasma samples from individuals who had a primary Den1 infection several months ago ($n = 5$) (2 representative samples depicted) (b), from individuals with an acute secondary infection with Den2 and known to have had a primary infection with Den1 ($n = 5$) (2 representative samples depicted) (c), and from individuals with an acute secondary infection with Den1 and known to have had a primary infection with Den2 ($n = 4$) (2 representative samples depicted) (d). (e) Enhancement assays were performed over the acute time course of an individual with Den2 and known to have had primary Den1 exposure. The number of days since defervescence from day 0 (d0) to day 213 (d213) are indicated after the patient identification.

relationship between the avidity of the antibody response and enhancing activity, suggesting that original antigenic sin may also influence ADE.

Anti-ED3 responses contribute little to neutralizing and enhancing activity. In mice, ED3 is a known target of neutralizing antibodies and so has been proposed as a target of immunization studies. We were therefore interested to investigate the contribution of anti-ED3 antibodies to neutralizing and enhancing activity in the human response. PCS and a selection of convalescent primary and secondary plasma samples were depleted of ED3 binding antibodies by incubation with agarose beads coated with ED3(1 and 2) or no protein as a control. To ensure that the His-tagged protein had completely depleted all anti-ED3 antibodies, we performed an ELISA with the untagged protein.

Following depletion, the neutralizing and enhancing activities of the plasma sample were determined. There was no reduction in neutralizing activity on Vero or 293T cells (data not shown), and this is in agreement with Wahala et al., who

showed that anti-ED3 antibodies did not contribute to neutralization in human sera (66). Interestingly, considering the highly cross-reactive nature of anti-ED3 antibodies in humans, depletion of anti-ED3 antibodies also had no effect on the ability of plasma samples to enhance Den2 infection in U937 cells (Fig. 5a to c). Furthermore, depletion in PCS did not affect activity on primary monocytes (Fig. 5d). Such measurements on monocytes are informative because they can be infected in the presence or absence of antibody, meaning that the full scope of both neutralization and enhancement is observed in one assay.

DISCUSSION

Original antigenic sin was first described in 1953 from observations on immune responses to influenza viruses (10, 15). Following on from this, in his “disquisitions on original antigenic sin,” Fazekas de St. Groth made detailed observations on children vaccinated with two strains of influenza, who by virtue

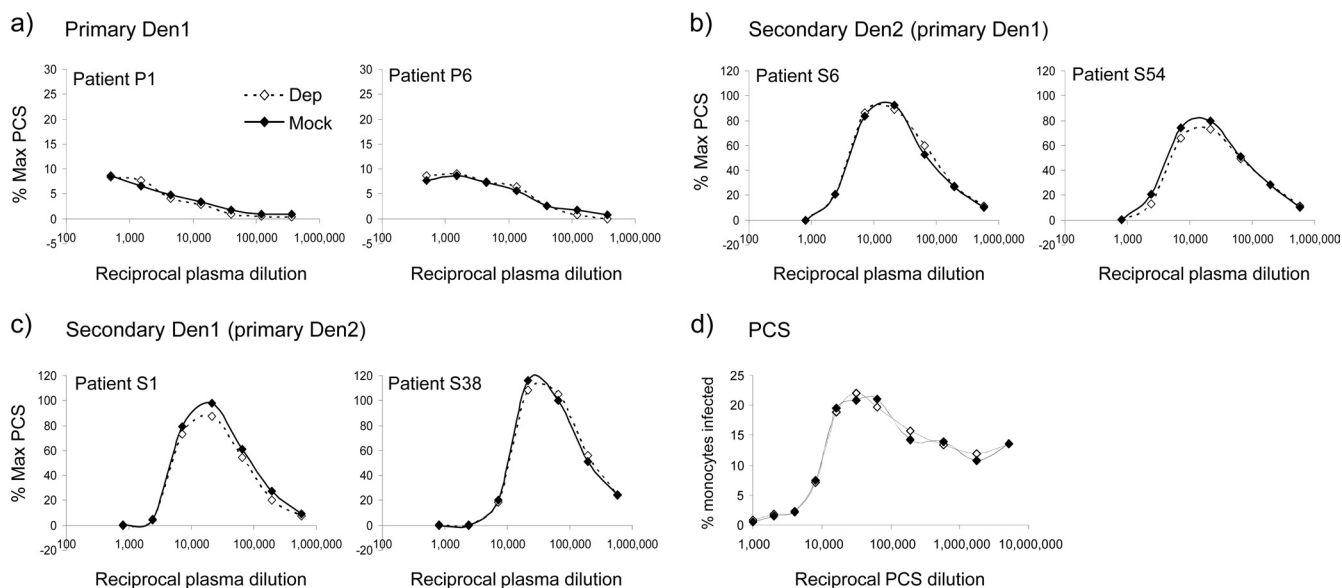


FIG. 5. Depletion of anti-ED3 antibodies from human plasma has no effect on enhancing activity. PCS and a selection of convalescent plasma samples were depleted completely of anti-ED3 antibodies. Samples were diluted and incubated with either His-tagged recombinant ED3(1 and 2), enabling pulldown with Ni^{2+} -coated agarose beads (Dep) or with no protein (beads-only, mock control). The depleted samples were then tested for enhancement of Den2 infection on U937 cells (a to c) or primary monocytes (d). (a to c) Plasma samples were tested from individuals with primary Den1 infection ($n = 5$) (2 representative samples depicted) (a), with a secondary Den2 infection and known to have had a primary Den1 infection ($n = 5$) (2 representative samples depicted) (b), or with a secondary Den1 infection and known to have had a primary infection with Den2 ($n = 4$) (2 representative samples depicted) (c). The percentages of cells infected were normalized to the maximum level of infection observed with a nondepleted PCS control. (d) PCS, depleted of anti-ED3 antibodies, was tested for its ability to both neutralize and enhance Den2 infection on primary monocytes. In the absence of serum, 13.7% infection was observed.

of their age could have been exposed to only one of these strains by natural exposure (13). Upon heterologous vaccination, fully cross-reactive antibodies were generated against the vaccinating strain and previously encountered strain. Despite recent reports corroborating these findings (34), there have been some studies with influenza vaccination that have questioned the significance or even the presence of original antigenic sin (21, 70).

The exact mechanisms of original antigenic sin are not fully understood. The degree of antigen similarity will clearly influence the ability of a single antibody to cross-react. It has been suggested that a difference of less than approximately 33 to 42% will result in original antigenic sin (6, 11). Interestingly, dengue antigens show approximately 30% amino acid difference between the serotypes, leaving them in a prime position for such a phenomenon. It is also suggested that cross-reactive memory responses are preferentially activated over naïve cells, as memory cells require a lower level of activation to proliferate (34). Furthermore, memory cells may outnumber naïve cells meaning that, when antigen levels are low, they could outcompete naïve cells for antigen. In addition, it appears that the vaccine formulation may also have a bearing on the effect (34).

Original antigenic sin in dengue was first described by Halstead in 1983 (26) and forms the basis for serological efforts to untangle the sequence of infection in secondary cases. More recently, original antigenic sin has also been described for dengue-specific T-cell responses (49). In this study, we have taken a close look at antibody responses to ED3 in both primary and secondary infections. Dengue seems to be an ex-

treme example of original antigenic sin, where the secondary response is entirely constructed from antibody that cross-reacts with previously encountered virus. Furthermore, these antibodies can be competed by 20- to 10,000-fold less antigen from the putative primary infecting serotype, indicating higher avidity for these antigens than for the secondary infecting virus.

Original antigenic sin has the advantage that a response can be rapidly mobilized from memory. However, the downside is that in some cases, such as dengue, the response is dominated by inferior-quality antibody. In influenza, original antigenic sin has been shown to reduce the effectiveness of vaccination (13, 34, 51). In dengue, the effect of original antigenic sin has considerable bearing on vaccine strategies. Once a response has been established, it is unlikely that repeat boosting will be able to change its scope, meaning that balanced responses against the four virus serotypes will need to be established with the first vaccine dose.

In this report, we have also shown that the primary infecting serotype, and thus antibody avidity, influences antibody-dependent enhancement in subsequent infections. The balance between neutralization and enhancement will likely determine the susceptibility of an individual to an infection and, by driving higher virus replication, may expose some individuals to the risk of developing DHF. An association between higher virus loads and DHF has been demonstrated (63), and we believe that the mobilization of a poor-quality antibody response in the early stages of secondary dengue infection may allow further amplification of virus replication via ADE.

In mice, some antibodies generated following infection with flaviviruses react to ED3 and show potent neutralization of

infection (18, 19, 27, 42, 43, 52, 61). These observations have generated interest in the use of ED3 as an immunogen for subunit dengue vaccines, and several flavivirus anti-ED3 antibodies have been suggested as potential therapeutics (5, 33, 50, 55, 60). It has recently been reported that some anti-ED3 neutralizing monoclonal antibodies do not neutralize all strains within a given serotype to equal levels (5, 55, 60, 65), meaning that therapeutic antibodies (Abs) will need to be carefully chosen.

In the experiments described here, we used prototypic dengue strains for the four serotypes and did not attempt to match to the virus causing each secondary infection. Nevertheless, the results on polyclonal human sera were clear, showing very high-level cross-reactivity during secondary infection. It remains possible that comparing a panel of ED3 strains from a given serotype in competition assays may allow a more precise description of the primary infecting virus beyond just its serotype.

ED3 has two potential attractions as an immunogen. First, antibodies directed to this domain are frequently of high avidity and show high neutralizing capacity for dengue virus compared to antibodies directed to other regions of the envelope protein (60). Second, ED3 is a target of serotype-specific mouse monoclonal antibodies (5, 18, 42, 45, 53, 55, 60, 61). Initially, it was thought that antibodies against this domain had limited cross-reactivity between the 4 serotypes, suggesting a reduced risk of ADE following vaccination. Recent reports in macaques (30) and humans (44, 66) also suggest that the primary ED3 response is relatively serotype specific. However, it has since been recognized that there are indeed cross-reactive mouse monoclonal antibodies against this domain (19, 43, 46, 52, 55, 60, 61), even though neither the binding avidities nor the critical binding amino acids need be the same between serotypes (46). Furthermore, while cross-reactive ED3 antibodies can be strong neutralizers (52, 55, 60), they are generally weaker neutralizers compared to their serotype-specific counterparts (19, 61), meaning that the potential for ADE remains significant.

In this study of the human serological response to dengue, we demonstrate a low to moderate level of cross-reactivity in the ED3 response following primary infection, although the highest response was mostly indicative of the infecting serotype. Subsequently, however, during a heterotypic secondary infection, this low-level cross-reactivity was dramatically boosted to the extent that 100% of antibody was cross-reactive.

Cross-reactive mouse monoclonal antibodies have been shown to bind with the highest avidity to ED3 serotype 1 (19, 64), and following ED3 immunization in mice, anti-ED3(1) polyclonal responses are also often the strongest (37). While these data correspond well with the high ED3(1) binding titers that we observed during secondary infections, we did not see a bias toward this serotype in our polyclonal competition avidity ELISA. It has also been reported that cross-reactive monoclonal antibodies bind poorly to ED3 from serotype 4 (19, 64). Consistent with this, following primary dengue infections in humans, we found reduced levels of seropositivity against ED3(4) compared to the remaining serotypes. This may be a consequence of the slight structural differences in the ED3 of this serotype (64).

In humans, it has recently been reported that anti-ED3 an-

tibodies constitute a relatively minor proportion of the response against dengue (8, 66), and it appears that a major part of the anti-envelope antibody response targets the fusion loop (8, 40). Corresponding with this, the anti-ED3 response seems to contribute little or nothing to neutralization in humans, as we demonstrate here in monocytes and as reported by Wahala et al. (66). The limited contribution of the anti-ED3 response to neutralization in a natural infection does not however mean that inducing a high-level response by vaccination would fail, and such vaccination strategies should not be discounted.

Finally, the ELISA competition assay we describe here may well provide an alternative to neutralization assays to predict the sequence of dengue infection. The technique is fast, does not require infection-containment facilities, and yields results comparable to the results of neutralization assays. Indeed, in a number of cases, neutralization results are complex and do not allow the diagnosis of the primary infection, whereas the competition ELISA usually gives clear definition of the avidity response.

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